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The skin provides a cover for the body, and serves as an immune competent organ. This project sought to determine whether the skin also has a neuroendocrine function. I have demonstrated that the majority of the cells in the epidermis, e.g. keratinocytes and the bone marrow derived Langerhans cells express the mRNA for proopiomelanocortin. Furthermore, I have shown that keratinocytes do not demonstrate detectable opiate receptors on their cell surface, but do express the sigma receptor. The proliferation of keratinocytes is not influenced by nerve growth factor. Finally, I have shown that keratinocytes secrete IL-6 but, contrary to a recent report in the literature, recombinant IL-6 does not stimulate the growth of normal human keratinocytes.

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FINAL REPORT

The Relationship of Stress and Susceptibility to Infections in the Skin

ONR CONTRACT N 00014-87-K-0216 P00003

Since last September when I received ONR funding, I have made considerable progress in linking the skin, i.e. the cells of the epidermis, to the neuroendocrine system and have provided evidence for the possibility that the skin is not only an immunologic organ but also a neuroendocrine organ. Thus, in collaboration with Simon Lee, Syntex Corp., I have shown that both Langerhans cells (LC) and keratinocytes synthesize the mRNA for proopiomelanocortin (POMC). (The presence of POMC mRNA in unstimulated LC and unstimulated keratinocytes was documented using a newly synthesized, more specific radioactive probe subsequent to the submission of the enclosed abstract.) LC are bone marrow derived immune competent cells with macrophage-like function(s). These new data provide a scientific basis for a link between the immune and neuroendocrine systems in the skin and thus may lead to the discovery of pathophysiologic mechanisms which underlie certain stress related skin diseases such as atopic dermatitis, herpes simplex and psoriasis.

EXPERIMENTAL STUDIES

The Presence of mRNA for Proopiomelanocortin (POMC) in LC

Epidermal cell suspensions were separated into LC enriched and depleted fractions by the panning method. Both fractions were incubated overnight plus or minus either phorbol myristate acetate (PMA) (10^{-7} M) or lipopolysaccharide (LPS) (20 ug/ml) (see attached abstract). (All of these experiments were performed in collaboration with Dr. Simon Lee, Syntex Corp., Palo Alto, CA).

Initial experiments demonstrated that PMA stimulated the expression of the POMC mRNA in human LC. Subsequent experiments using a more specific probe demonstrated that unstimulated LC as well as unstimulated keratinocytes also express mRNA for POMC (Fig. 1).

LC Synthesize IL-1 as well as the mRNA for IL-1

The supernatant media of LC cultured for 2 days were harvested and immunoassayed for IL-1. No IL-1 beta protein was detected in the supernatant medium. However, both IL-1 alpha and beta was detected in the cell lysates. The level of protein synthesized was greatly increased by incubation with PMA. Thus, IL-1 protein is produced but not secreted by LC. In the same experiment, we assayed the mRNA for both IL-1 alpha and beta (Fig. 2). No IL-1 mRNA synthesis was detectable unless the cells were incubated with PMA (10^{-7} M) which stimulated synthesis of both IL-1 alpha and beta mRNA. Muramyl dipeptide (MDP) alone had no effect.

LPS (10 ug/ml) and ionomycin (0.1 uM) alone or in combination did not stimulate expression of either IL-1 mRNA. As would be predicted from the protein synthesis data, IL-1 beta mRNA was more highly expressed than the mRNA for IL-1 alpha. IL-1 beta did not stimulate synthesis of its own mRNA.

The Effect of Dexamethasone (Dex) on IL-1 beta mRNA Expression in LC

Since corticosteroids are potent anti-inflammatory agents, we examined the effect of Dex (10 uM) on IL-1 beta mRNA expression. Dex had no effect on the expression of IL-1 beta mRNA.

The Effect of Various Peptides on Sythesis of IL-1 mRNA by LC

To determine whether we could identify a "physiologic" protein which could induce IL-1 mRNA in LC, we incubated the cells with a variety of substances including IGF-1 (100 ng/ml), G/M-CSF (10 ng/ml), IL-6 (50 U/ml), none of these peptides induced mRNA for IL-1.

The Effect of Cycloheximide (CHX) on the Sythesis of IL-1 mRNA

To determine whether LC synthesize a protein which inhibits expression of mRNA for IL-1, we incubated the LC with CHX (10 ug/ml). The addition of CHX (without other activators) did not allow the expression of IL-1 mRNA. Thus, LC do not produce a protein which inhibits the expression of mRNA for IL-1.

The Effect of IL-6 on Keratinocyte Proliferation in Vitro

It has been reported recently that IL-6 stimulates the proliferation of human keratinocytes in vitro (Grossman, R., 1989). The authors' implication was that since IL-6 mRNA and protein are strongly expressed in psoriatic lesional skin and not in control skin, the IL-6 accounts for the increased proliferation of keratinocytes in this disease process.

I have had a great deal of experience with use of the Clonetics medium, both keratinocyte growth medium (KGM) and keratinocyte basal medium (KBM), for culturing keratinocytes (Morhenn, et al, 1989). During the course of these experiments, I documented that very small amounts of BSA (0.004%) when added to KGM or KBM will stimulate keratinocyte growth. Subsequent to reading the paper by Grossman, et.al, I telephoned Genzyme Corp. and asked whether they produce IL-6 which is free of a protein carrier. I was told that all the IL-6 sold by the Company comes diluted in 0.1% bovine serum albumin (BSA). Thus, I began to doubt the conclusions in the Grossman paper. It was my view that the stimulation of keratinocyte documented in that paper is due to the small amount of BSA (0.001%) added, not to the IL-6 itself. Therefore, we have added recombinant IL-6 (rIL-6) (R & D Systems) which is carrier free to keratinocytes cultured in both KBM and KGM plus BPE and have seen no increase in the numbers of cells/plate in rIL-6 treated vs. control cultures. The same result was obtained in a second separate experiment.

To further document the effect of rIL-6 on keratinocytes we assayed the incorporation of tritiated thymidine ($^3\text{H-T}$) into keratinocyte DNA 24 hrs. after the addition of 10 ng/ml rIL-6 as described by Grossman, et al, 1989. The cultures were pulsed with the $^3\text{H-T}$ for 4 hrs. before harvesting and counting in a liquid scintillation counter. Cells cultured in KGM, KBM or KGM + bovine pituitary extract (BPE) did not show significantly increased $^3\text{H-T}$ incorporation in the presence of rIL-6 (Table).

We have documented that our IL-6 is active because:

- 1.) The rIL-6 diluted from the stock vial received from the company supports the growth of B-9 cells, a murine hybridoma cell line which is dependent on IL-6 for growth/survival.
- 2.) Supernatant medium containing rIL-6 used for the keratinocyte cultures was removed after 48 hrs. and used in the B-9 stimulation assay. This rIL-6 containing medium stimulated $^3\text{H-T}$ incorporation (22,057 dpm) vs. control supernatant culture medium without rIL-6 (2,553 dpm).

My conclusion from these experiments is that IL-6 itself does not stimulate keratinocyte growth and therefore probably does not play a direct role in the keratinocyte hyperproliferation seen in psoriasis.

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Keratinocyte Secretion of IL-6 Varies with Cell Density

Keratinocytes secrete IL-6 as measured by the B-9 proliferation assay. We have shown that the secretion of IL-6 is dependent on the rate of proliferation of the cells. Thus, confluent keratinocyte monolayers make less IL-6 (0.0035 fg/cell/24 hrs.) than do proliferating cells (0.045 fg/cell/24 hrs.).

Insulin-Like Growth Factor (IGF) Receptors on Keratinocytes, LC and SCL-1 Cells

We have demonstrated the presence of receptors for IGF/somatomedin-C on human keratinocytes and the transformed squamous cell carcinoma cell line, SCL-1. IGF-1 and IGF-2 were growth stimulatory for both cell types. The LC also demonstrated the IGF-1 and -2 as well as insulin receptors, with ^{125}I -IGF-2 giving the strongest binding. Finally, SCL-1 cells demonstrated membrane associated IGF binding proteins.

The Effect of Nerve Growth Factor and Beta-Endorphin on Keratinocyte Proliferation

I have examined whether keratinocyte proliferation is affected by nerve growth factor (a generous gift of K. Nikolics, Genentech Corp.). This protein did not affect keratinocyte growth at a wide range of concentrations (1-1,000 U/ml) tested. I also have investigated the effect of beta-endorphin on the growth of keratinocytes. The proliferation of keratinocytes cultured in keratinocyte growth medium (KGM) (Clonetics, Corp., San Diego, Ca) with and without bovine pituitary extract (BPE) was not affected by beta-endorphin. In concurrent experiments, I have examined the question whether keratinocyte cultured in vitro express receptors for opiates (see above). In keeping with and in support of the finding that keratinocytes do not express opiate receptors was our demonstration that the growth of keratinocytes is not affected by beta-endorphin.

Fc Receptor Expression by Keratinocytes Is Not Inducible by Gamma Interferon

Gamma interferon (gamma IFN) increases the expression of the type I Fc receptor for IgG in U937 cells (Anderson et al, 1986). The monoclonal antibodies (mAbs) 22, 32, IV.3, and 248 (kindly provided by Dr. Guyre, Dartmouth, NH), specifically bind to the Fc receptor(s). Since gamma IFN induces expression of various markers on human keratinocyte, (eg. HLA-DR, intercellular adhesion molecule), I asked whether gamma IFN could induce type I Fc receptor expression on keratinocytes. The keratinocytes were incubated with gamma IFN (100 U/ml) for 48 hrs. in one experiment and 72 hrs. in a second experiment. Then, the keratinocytes were labeled with mAb 22, 32, IV.3 or 248 for 30 min., washed and labeled with fluorescein isothiocyanate conjugated goat anti-mouse IgG (G/M-FITC) for another 30 min. As a positive control, aliquots of these keratinocytes treated with gamma IFN or untreated were stained with mAb against HLA-DR followed by G/M-FITC. All the cells were fixed with formaldehyde and analyzed using a fluorescence activated cell sorter. The gamma IFN treated keratinocytes showed no staining with the anti-Fc receptor antibodies compared to untreated controls. By contrast, the keratinocytes did express HLA-DR after the gamma IFN treatment. Another set of positive controls showed that gamma IFN did increase the expression of the type I Fc receptor in U937 cells under the experimental conditions used. Thus, we have been unable to induce the expression of the type I Fc receptor with gamma IFN in human keratinocytes.

Bradykinin's (BK) Effect on Normal and Transformed Human Keratinocytes

In collaboration with Randy Johnson (Genentech, Corp.), I have shown that BK, a nonapeptide, stimulates phosphoinositol (PI) synthesis in both normal and transformed human keratinocytes. Diacylglyceride (DAG) also is stimulated in these cells by BK. The response of non-transformed keratinocytes to BK (2-100 nM) is stronger than that seen in SCL-1 cells, a transformed squamous cell carcinoma cell line. Thus, normal keratinocytes stimulated with 0.1 μ M BK showed a 2.9 fold stimulation of inositol (1,4,5) P_3 (ratio of treated over control) whereas SCL-1 cells showed a ratio of 1:1. It has been documented that PI's trigger Ca^{++} uptake by various cells. Therefore, we examined the effect of BK and other cytokines on intracellular Ca^{++} levels. Thirty seconds after addition of BK (2 nM) to SCL-1 cells, intracellular calcium levels increase dramatically compared to control levels. A further thirty sec. later, the intracellular Ca^{++} concentration has decreased virtually to baseline levels and another 30 sec. later the cells have again increased their intracellular Ca^{++} a small amount over baseline in an oscillating fashion. Normal keratinocytes showed a similar and more dramatic response to BK.

Studies to Determine the Presence of Sigma and Opiate Receptors on Keratinocytes

In preliminary experiments performed in collaboration with Dr. L. Toll, SRI, Menlo Park, CA, we have demonstrated sigma receptors on human keratinocytes. These studies were performed using 3H -propyl-3 (3-hydroxyphenyl) piperidine as the ligand. By contrast, keratinocytes do not express opiate receptors.

INVENTIONS: None

PUBLICATIONS AND REPORTS:

1. I have completed two papers, one on the subject of LC IL-1 beta synthesis and mRNA expression and a second paper on the detection of IL-1 (mainly alpha) in keratinocytes. Titles below, copies enclosed.

a) Morhenn VB, Lee SW, Ilnicka M, Eugui EM: Activated human Langerhans cells express mRNA for interleukin-1 alpha and interleukin-1 beta and produce these cytokines but do not secrete them. Submitted 1990.

b) Lee SW, Morhenn VB, Ilnicka M, Eugui EM, Allison AC: Autocrine stimulation of interleukin-1 alpha and transforming growth factor alpha production in human keratinocytes and its antagonism by glucocorticoids. Submitted 1990.

2. Three papers are in press; titles below:

a) Kilmer SL, Berman B, Morhenn VB: Eruptive seborrheic kerratoses in a young woman with acromegaly. J Am Acad Dermatol.

b) Morhenn VB: "Pemphigus an alternative approach to treatment." Letter. Arch Dermatol.

c) Neely EK, Morhenn VB, Hintz RL, Wilson DM, Rosenfeld RG: Insulin-like growth factors are mitogenic for human keratinocytes and a squamous cell carcinoma. J Investig Dermatol.

3. An abstract of our POMC mRNA data was recently published and presented in poster form at the national meeting of the Soc. Investig. Dermatol. Title below, copy enclosed. A second abstract unrelated to the work supported directly by this contract was also published. Title below, copies enclosed.

a) Morhenn VB, Stage K, Lee S. (1990) Activated human Langerhans cells contain mRNA for proopiomelanocortin. *J. Invest. Dermatol.* 94:557.

b) Morhenn VB, King KL, Johnson, RM. (1990) Dissociation of inositol lipid hydrolysis from cell proliferation following treatment with growth factors and bradykinin in human keratinocytes. *J. Invest. Dermatol.* 94:557.

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Bieber T, Rieger A, Neuchrist C, Prinz JC, Rieber EP, Boltz-Nitulescu G, Scheiner O, Kraft D, Ring J, Stingl G. *J Exp Med* 170:309-314, 1989.

Grossman RM, Krueger J, Yourish D, Granelli-Piperno A, Murphy DP, May LT, Kupper TS, Sehgal PB, Gottlieb AB. *Proc Natl Acad Sci USA* 86:6367-6371, 1989.

Morhenn VB, Wastek GJ, Cua A, Mansbridge JN. *J Invest Dermatol* 89:121-125, 1989.

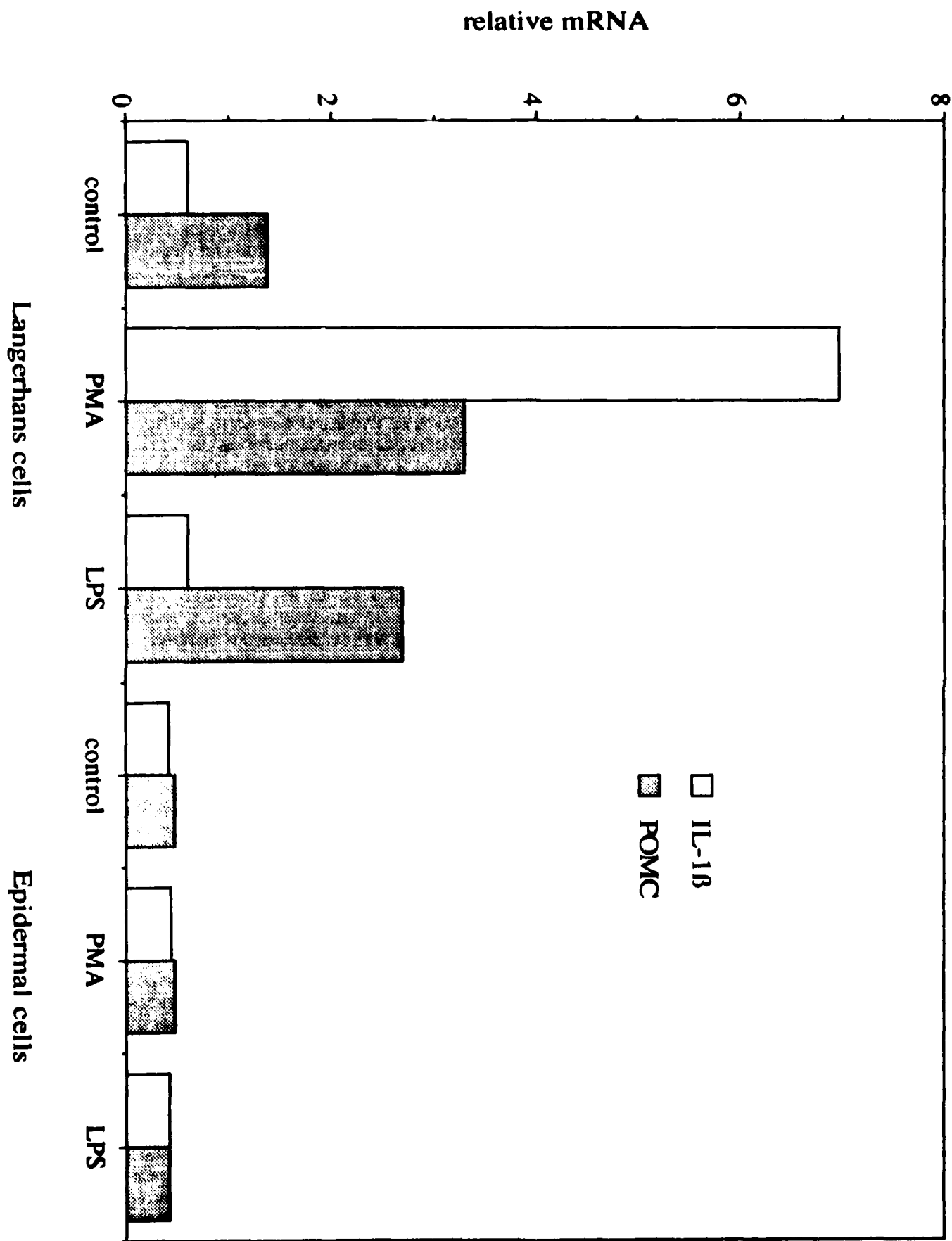


Fig. 1

FIGURE 2

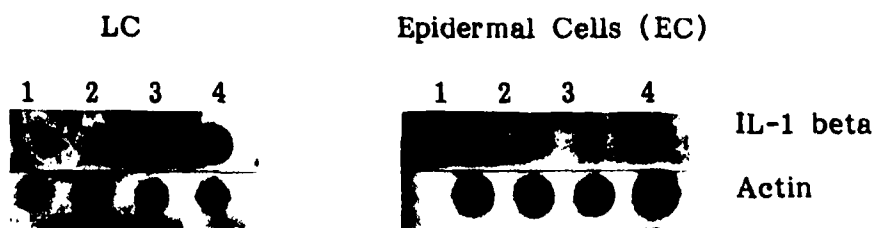


Figure Legend. The cells were incubated overnight with various substances: 1) control, 2) interferon alpha (20 ng/ml), 3) IL-1 alpha (27 ng/ml), 4) PMA (100 nM) and the RNA extracted using RNazol. Dot blots were prepared and probed with the indicated radioactive cDNAs (IL-1 beta, actin). The IL-1 beta blot was exposed for two days. The beta-actin blot was exposed for four days. For LC, the IL-1 beta mRNA was detected only in PMA-treated cells.